

20030104608. 09 Mar 93. 05 Jun 03. METHOD, COMPOSITION AND DEVICE FOR REMOVING OXYGEN FROM SOLUTIONS CONTAINING ALCOHOLS AND/OR ACIDS. COPELAND, JAMES C., et al. 435/262; C07C001/00.

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☐ 2. 20020045245. 19 Mar 01. 18 Apr 02. Apparatus and method for growing anaerobic microorganisms. Copeland, James C., et al. 435/305.3; 435/303.2 C12M001/22.

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☐ 3. 6429008. 19 Mar 01; 06 Aug 02. Apparatus and method for growing anaerobic microorganisms. Copeland; James C., et al. 435/303.2; 422/102 435/305.4 435/307.1 435/801. C12M001/00.

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☐ 4. 6204051. 28 May 99; 20 Mar 01. Apparatus and method for growing anaerobic microorganisms. Copeland; James C., et al. 435/305.4; 435/288.3 435/303.2 435/801. C12M001/22.

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☐ 5. 5955344. 03 Nov 97; 21 Sep 99. Apparatus and method for growing anaerobic microorganisms. Copeland; James C., et al. 435/243; 435/288.3 435/303.2 435/305.4 435/307.1 435/395 435/420 435/801. C12N001/00.

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☐ 6. 5830746. 04 May 94; 03 Nov 98. Apparatus and method for growing anaerobic microorganisms. Copeland; James C., et al. 435/243; 435/303.2 435/305.4. C12M001/00.

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☐ 7. 5482860. 20 Apr 93; 09 Jan 96. Apparatus for continuously removing oxygen from fluid streams. Copeland; James C., et al. 435/293.1; 435/297.1 435/813. C12M001/40.

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☐ 8. 5432083. 19 Feb 93; 11 Jul 95. Enzymatic method for removing oxygen from oils and fats. Copeland; James C., et al. 435/271; 426/417 426/601 435/262 435/317.1. C12S003/00 C11C001/00 C12N001/00 A23L003/3463.

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☐ 9. 5240853. 07 Mar 89; 31 Aug 93. Apparatus and method for continuously removing oxygen from fluid streams using bacterial membranes. Copeland; James C., et al. 435/262; 435/820. C12N011/18.

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☐ 10. 4996073. 29 Aug 89; 26 Feb 91. Method and composition for removing oxygen from solutions containing alcohols and/or acids. Copeland; James C., et al. 426/487; 426/541 426/544 426/592 435/161 435/262 435/264 435/801 435/820. C12G001/00 C12H001/00.

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☐ 11. WO009530738A1. 04 May 95. 16 Nov 95. APPARATUS AND METHOD FOR GROWING ANAEROBIC MICROORGANISMS. COPELAND, JAMES C., et al. C12M001/00; C12M001/16 C12M001/18 C12M001/20.

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☐ 12. WO008804319A1. 04 Dec 87. 16 Jun 88. MATERIAL AND METHOD FOR PROMOTING GROWTH OF ANAEROBIC BACTERIA. ADLER, HOWARD I., et al. 435/253.6. C12N001/20; C12N001/00.

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☐ 13. US 6429008B. Culture dish assembly for growing anaerobic microorganisms, comprises first and second dishes having wall and side wall. ADLER, H I., et al. C12M001/00 C12M001/22.

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☐ 14. US 6204051B. Culture dish assembly for automated preparation of e.g. agar, necessary for commercial production, includes dishes that form non-sealed assembly in first orientation and closed headspace in second orientation. ADLER, H I., et al. C12M001/22.

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☐ 15. US 5830746A. Appts. for growing anaerobic microorganisms - with top and bottom dish and

intervening sealing ring contacting media surface on inversion of appts. to trap anaerobic gas in head-space. ADLER, H I, et al. C12M001/00 C12M001/16 C12M001/18 C12M001/20 C12M001/22 C12N001/00.

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☐ 16. WO 8804319A. Nutrient medium for promoting growth of anaerobic bacteria - includes a hydrogen donor and sterile membrane fragments derived from mitochondria having membranes contg. electron transfer system. ADLER, H I, et al. C12N001/20.

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Term	Documents
(43 NOT 42).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	16
(L43 NOT L42).PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD.	16

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12M 1/00, 1/16, 1/18, 1/20</b>	<b>A1</b>	(11) International Publication Number: <b>WO 95/30738</b> (43) International Publication Date: 16 November 1995 (16.11.95)
(21) International Application Number: <b>PCT/US95/05571</b> (22) International Filing Date: <b>4 May 1995 (04.05.95)</b> (30) Priority Data: <b>08/237,773 4 May 1994 (04.05.94) US</b> (71) Applicant: <b>OXYRASE, INC. [US/US]; P.O. Box 1345, Mansfield, OH 44901 (US).</b> (72) Inventors: <b>COPELAND, James, C.; 289 N. Countryside Drive, Ashland, OH 44805 (US). ADLER, Howard, I.; 128 Indian Lane, Oak Ridge, TN 37830 (US). SPADY, Gerald, E.; 111 South Hollywood Circle, Oak Ridge, TN 37830 (US).</b> (74) Agent: <b>KLEIN, Richard, M.; Fay, Sharpe, Beall, Fagan, Minnich &amp; McKee, Suite 700, 1100 Superior Avenue, Cleveland, OH 44114-2518 (US).</b>	(81) Designated States: <b>AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, MX, NO, NZ, PL, PT, RO, RU, SG, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</b>  <b>Published</b> <i>With international search report.</i>	
(54) Title: <b>APPARATUS AND METHOD FOR GROWING ANAEROBIC MICROORGANISMS</b> (57) Abstract <p>An apparatus for growing anaerobic microorganisms is provided having a dish top that contains a sealing ring upon which the media surface in the dish bottom rests when the apparatus is inverted. The contact between the sealing ring and the media surface forms a seal that traps the gas in the headspace between the media surface and the inside of the dish top. An oxygen reducing agent can also be incorporated into the media together, in some instances, with a substrate which react with oxygen in the media and with oxygen in the headspace thereby creating an environment suitable for growing anaerobic, microaerophilic and facultative anaerobic microorganisms.</p>		

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L32: Entry 3 of 8

File: PGPB

Apr 18, 2002

DOCUMENT-IDENTIFIER: US 20020045245 A1

TITLE: Apparatus and method for growing anaerobic microorganisms

Summary of Invention Paragraph:

[0003] The present invention relates to an apparatus and method for growing anaerobic microorganisms. The apparatus is comprised of a specially designed culture dish which can be reconfigured such as by inverting the dish to produce an anaerobic environment. An oxygen reducing agent such as a biocatalytic oxygen reducing agent can also be incorporated into the media present in the apparatus together, in some circumstances, with a substrate. The biocatalytic oxygen reducing agent and the substrate present in the media react with oxygen enclosed in the culture dish to create an environment suitable for growing and maintaining anaerobic microorganisms.

Summary of Invention Paragraph:

[0027] The present inventors have designed a novel culture apparatus or dish in order to eliminate many of the difficulties observed in the prior art. It has been found that the use of the new culture dish (i.e., "OxyDish.TM.") together with an oxygen reducing agent (preferably a biocatalytic oxygen reducing agent) and, in some instances, a substrate, produces a controlled, self-contained environment for isolating, enumerating, identifying and growing facultative aerobes, microaerophiles and anaerobes. The use of the specially designed culture dish along with an oxygen reducing agent makes possible the design and function of a culture dish that utilizes some features of the Brewer Lid, but overcomes its limitations and makes possible novel and improved characteristics.

Summary of Invention Paragraph:

[0028] In this regard, the present invention is directed to a specifically designed culture dish with a dish top or cover that contains a sealing ring on the inside upon which the solid media surface in the bottom dish rests when the dish is inverted to form a media-ring seal. The seal so formed traps the gas in the headspace between the media surface and the inside of the dish top or cover. In addition, an oxygen reducing agent, such as a biocatalytic oxygen reducing agent, can be incorporated into the media present in the culture dish together, in some instances, with a substrate which reacts with oxygen in the media and the headspace to create an environment suitable for growing anaerobic microorganisms.

Summary of Invention Paragraph:

[0029] The preferred biocatalytic oxygen reducing agent (see "A Novel Approach to the Growth of Anaerobic Microorganisms" of Adler, et al., Biotechnol. Bioeng. Symp. 11, J. Wiley & Sons, New York, 1981, p. 533 and U.S. Pat. No. 4,476,224 issued Oct. 9, 1984 to Adler entitled "Material and Method for Promoting the Growth of Anaerobic Bacteria") utilized in the invention is comprised of oxygen scavenging membrane fragments which contain an electron transport system which reduces oxygen to water in the presence of a hydrogen donor. These oxygen scavenging membrane fragments can be derived from the cytoplasmic membranes of bacteria (U.S. Pat. No. 4,476,224) and/or from the membranes of mitochondrial organelles of a large number of higher non-bacterial organisms. Other known biocatalytic oxygen reducing agents such as glucose oxidase, alcohol oxidase, etc. can also be utilized.

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L13: Entry 63 of 64

File: USPT

Nov 8, 1983

DOCUMENT-IDENTIFIER: US 4414334 A

TITLE: Oxygen scavenging with enzymesBrief Summary Text (24):

As a catalyst, the enzyme is not consumed but functions continuously as long as oxygen and aqueous alcohol are both present. It is noteworthy that reaction ceases if ambient oxygen is depleted but is resumed if more oxygen is introduced into the system. Therefore, additional oxygen is continuously removed and the enzymes, unlike conventional oxygen scavengers, are not used up. Thus only a relatively small amount of enzyme is required to catalyze the removal of a relatively large amount of oxygen from aqueous alcohol mixtures.

Brief Summary Text (27):

In applications where the H.sub.2 O.sub.2 by-product is preferred such as in selected drilling muds, the purified alcohol oxidase is used. The generated H.sub.2 O.sub.2, e.g., can react with the carboxymethylcellulose or starch in drilling muds to improve the product. Another use of the purified alcohol oxidase is in waterfloods wherein the by-product H.sub.2 O.sub.2 functions as a biocide against downhole anaerobic microorganisms. In such applications a dual biocidal effect would be produced from the alcohol added, e.g., CH.sub.3 OH, and the by-product H.sub.2 O.sub.2 generated in-situ. The further injection of O.sub.2 downhole to produce more H.sub.2 O.sub.2 in-situ would provide additional biocidal effects.

Brief Summary Text (32):

Thus, the inventive deoxygenation systems are useful in decreasing or preventing the corrosion of corrosion-susceptible metals, particularly iron, which are in contact with closed circulating oxygen-containing water systems such as those passing through condensers, engine jackets, heat exchangers, evaporators, distribution systems, and the like as well as water storage tanks. The oxygen-scavenging systems thus help to avoid corrosion of metals commonly used in circulating water systems particularly ferrous metals including iron and steel, and also galvanized steel as well as non-ferrous metals including copper and its alloys, aluminum and its alloys, and brass. Of course, on a practical basis, it is not feasible to deoxygenate a cooling tower or evaporative condenser water due to constant re-saturation with air (oxygen).

Detailed Description Text (18):

The net effect of the reactions catalyzed by the enzyme combination of alcohol oxidase and catalase is the effective scavenging of free oxygen and the conversion of the by-product H.sub.2 O.sub.2 into water.

Detailed Description Text (21):

The enzyme-catalyzed deoxygenation systems described herein are operable over a pH range of 6 to 9 with an optimum pH range of 6.5 to 7.5. A temperature range of 0.degree. to 60.degree. C. is suitable with an optimum temperature range of about 40.degree. to 50.degree. C. The enzyme preparations can be stored indefinitely at 0.degree. C. without any appreciable loss of activity. The catalytic enzymes of the subject deoxygenation systems are active over a salinity range of 500 ppm total dissolved solids (TDS) to about 300,000 ppm TDS. In regard to stabilizers, 100 to 500 ppm formaldehyde or about 0.02 weight percent sodium azide is effective in

Detailed Description Text (42):

Detailed Description Text (43):

Detailed Description Text (46):

Detailed Description Text (67):

Detailed Description Text (68):

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a 20 hour period.

Detailed Description Text (70):

The alcohol oxidase system continues to scavenge oxygen effectively if the polymer solution is recontacted with oxygen. The alcohol oxidase-methanol system preferably also with catalase can be used in combination with the hydrosulfite system if desired to provide viscosity-stabilized aqueous fluids.

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## WEST Search History





DATE: Tuesday, October 25, 2005

Hide?	Set Name	Query	Hit Count
	<i>DB=USPT; PLUR=YES; OP=OR</i>		
<input type="checkbox"/>	L1	4808517.pn.	1
<input type="checkbox"/>	L2	L1 and particles	1
<input type="checkbox"/>	L3	L1 and (coli or bacteria or prokaryote or procaryote or escherichia)	1
<input type="checkbox"/>	L4	media or medium	768111
<input type="checkbox"/>	L5	L4 and azide	18300
<input type="checkbox"/>	L6	L5 and \$membrane	8186
<input type="checkbox"/>	L7	L6 and (anaerobic\$ or anaerobe or facultative\$)	294
<input type="checkbox"/>	L8	l4.clm.	215664
<input type="checkbox"/>	L9	L8 and \$azide.clm.	406
<input type="checkbox"/>	L10	L9 and membrane.clm.	20
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<input type="checkbox"/>	L13	L12 and (\$azide or \$cyanide)	64
<input type="checkbox"/>	L14	(\$oxidase same \$azide)	1684
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<input type="checkbox"/>	L18	L17 and oxyrase\$	37
<input type="checkbox"/>	L19	L18 and (cyanide or azide)	6
<input type="checkbox"/>	L20	membran\$ near5 (n3 or azide)	132
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<input type="checkbox"/>	L23	nan3 and oxyrase	0
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	L26 same (media or medium or growth or growing or isolation or isolating or		



<input type="checkbox"/>	L28	subculturing or sub-culture)	194
<input type="checkbox"/>	L29	l28 same (anaerobe or anaerobic or anerobically or anerobic or anerobe or anerobically or facultatively or facultative)	21
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<input type="checkbox"/>	L31	L30 and azide	5
<input type="checkbox"/>	L32	biocatalytic near oxygen	8
<input type="checkbox"/>	L33	electron near transport near inhibitor	79
<input type="checkbox"/>	L34	L33 same l11	2
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<input type="checkbox"/>	L36	L33 and oxyrase	2
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END OF SEARCH HISTORY

<b>#25 Search anaerobic cyanide culture</b>	14:30:15	<u>4</u>
<b>#24 Search anaerobic cyanide</b>	14:30:07	<u>4</u>
<b>#23 Search anoxic broth cyanide</b>	14:29:13	
<b>#22 Search anoxic broth azide</b>	14:29:02	
<b>#19 Search oxygen scavenging azide</b>	14:26:53	<u>2</u>
<b>#18 Search oxygen scavaging azide</b>	14:26:51	
<b>#17 Search oxygen scavaging azide growth medium</b>	14:26:46	
<b>#15 Search oxidase azide growth medium</b>	14:24:15	<u>2</u>
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<b>#7 Search oxidase azide anoxic</b>	14:17:26	
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<b>#1 Search anaselect azide</b>	13:31:58	<u>109</u>

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May 27, 1997

TITLE: Fermentor with vertical shaft

All bacteria, including both archaeobacteria and eubacteria, generally have more than one terminal oxidase (Anraku and Gennis, supra), and thus all except obligate anaerobes are potentially susceptible to DO.sub.2 instabilities upon culturing. Suitable bacteria for this purpose include aerobic and facultative anaerobic bacteria, whether archaeobacteria and eubacteria, especially eubacteria, and most preferably Enterobacteriaceae. Examples of useful bacteria include Escherichia, Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla, and Paracoccus. Suitable E. coli hosts include E. coli W3110 (ATCC 27,325), E. coli 294 (ATCC 31,446), E. coli B, and E. coli X1776 (ATCC 31,537). These examples are illustrative rather than limiting. Mutant cells of any of the above-mentioned bacteria may also be employed. It is, of course, necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, E. coli, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYA177, or pKN410 are used to supply the replicon.

It has been found herein that when the organism has previously been induced for the presence of menaquinone and the cytochrome d oxidase complex, it has the ability to scavenge low concentrations of oxygen but does so inefficiently. When sufficient oxygen is present, it would appear that this inefficient pathway is not allowed to function.

Doelle and Hollywood, "Transitional Steady-State Investigations during Aerobic-Anaerobic Transition of Glucose Utilization by Escherichia coli K-12" European Journal of Biochemistry 83:479-484 (1978).

Harrison and Loveless, "The Effect of Growth Conditions on Respiratory Activity and Growth Efficiency in Facultative Anaerobes Grown in Chemostat Culture" J. Gen. Microbiology 68:35-43 (1971).

Tamura-Lis and Webster, "Cyanide- and Carbon Monoxide-Resistant Mutants of Vitreoscilla: Altered Cytochromes and Respiratory Properties" Arch. Biochem. and Biophys. 244(1):285-291 (1986).

Tamura-Lis and Webster, "Respiration in Carbon Monoxide and Cyanide Resistant Strains of *Vitreoscilla*" Fed. Proc. (Ab. #2799) 41(3):749 (1982).

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L31: Entry 2 of 5

File: USPT

Jan 22, 1991

DOCUMENT-IDENTIFIER: US 4987076 A

TITLE: Uricase and a method for the preparation thereof

Detailed Description Text (49):

14. Growth in anaerobic culture medium: no

Detailed Description Text (51):

16. Growth at 55.degree. C. in a culture medium containing 0.02% sodium azide: no

Detailed Description Text (60):

As a result of the examination undertaken according to the method of classification described in Bergey's Manual of Determinative Bacteriology, 8th edition (1974) making reference to the above mentioned bacteriological properties of the microorganism, the subject microorganism of TB-90 was identified to belong to the genus of Bacillus. Although it may be a tentative conclusion derived from the comparison with known species belonging to the genus of Bacillus that the above described TB-90 can be either of Bacillus stearothermophilus, Bacillus coagulans and Bacillus brevis in respect of the temperature range for growth, this tentative conclusion is not supported due to the lack of motility in TB-90. Moreover, TB-90 can be differentiated from Bacillus stearothermophilus in respect of the ability of growth in a Sabouraud dextrose agar culture medium, from Bacillus coagulans in respect of the inability of growth in an anaerobic agar culture medium and in the presence of 0.02% of sodium azide and from Bacillus brevis in respect of the production of acid from xylose, no production of alkali in a V-P culture medium and inability of decomposing casein and tyrosine. In addition, uricase could not be produced when the culturing procedure described below was undertaken of the type strains including Bacillus stearothermophilus IAM 11001, 11002, 11003, 11004 and 12043, Bacillus coagulans IAM 1194 and Bacillus brevis IAM 1031 deposited at Institute of Applied Microbiology of Tokyo University.

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L32: Entry 1 of 8

File: PGPB

Jun 16, 2005

DOCUMENT-IDENTIFIER: US 20050130252 A1

TITLE: Stabilized susceptibility tests of aerobic pathogens

Detail Description Paragraph:

[0032] Adjuvant means a substance which enhances the effectiveness of medical treatment and further means a substance that may or may not have antimicrobial activity in and of itself, but in combination with an antibiotic in a sufficient amount, acts to stabilize and enhance susceptibility testing. Adjuvants include those selected from the group cysteine, thioglycolate, ascorbic acid, pyruvate and catalase. The methods and compositions described herein also include the use of adjuvants derived from the cytoplasmic membranes of microorganisms such as E. coli which act as stabilizing agents. OXYRASE.RTM. is the trademark for the cytoplasmic membranes of microorganisms such as E. coli marketed by Oxyrase, Mansfield, Ohio 44901, further known herein as Oxyrase.RTM. or Oxyrase.RTM. Enzyme System. Oxyrase.RTM. is also known as a biocatalytic oxygen reducing agent. Further, the adjuvants which are oxygen reducing agents and those that are derived from the cytoplasmic membranes of microorganisms such as E. coli which contains oxygen scavenging membrane fragments, can be used alone or in combination with other adjuvants.

Detail Description Paragraph:

[0066] The preferred Oxyrase.RTM. enzyme system utilized as an adjuvant in the invention is comprised of oxygen scavenging membrane fragments which contain an electron transport system which reduces oxygen to water in the presence of a hydrogen donor. These oxygen scavenging membrane fragments can be derived from the cytoplasmic membranes of bacteria and/or from the membranes of mitochondrial organelles of a large number of higher non-bacterial organisms. Other known biocatalytic oxygen reducing agents such as glucose oxidase, alcohol oxidase, catalase, etc. can also be supplemented or utilized in the present invention, although generally less preferably.

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L32: Entry 1 of 8

File: PGPB

Jun 16, 2005

DOCUMENT-IDENTIFIER: US 20050130252 A1

TITLE: Stabilized susceptibility tests of aerobic pathogens

Detail Description Paragraph:

[0032] Adjuvant means a substance which enhances the effectiveness of medical treatment and further means a substance that may or may not have antimicrobial activity in and of itself, but in combination with an antibiotic in a sufficient amount, acts to stabilize and enhance susceptibility testing. Adjuvants include those selected from the group cysteine, thioglycolate, ascorbic acid, pyruvate and catalase. The methods and compositions described herein also include the use of adjuvants derived from the cytoplasmic membranes of microorganisms such as E. coli which act as stabilizing agents. OXYRASE.RTM. is the trademark for the cytoplasmic membranes of microorganisms such as E. coli marketed by Oxyrase, Mansfield, Ohio 44901, further known herein as Oxyrase.RTM. or Oxyrase.RTM. Enzyme System. Oxyrase.RTM. is also known as a biocatalytic oxygen reducing agent. Further, the adjuvants which are oxygen reducing agents and those that are derived from the cytoplasmic membranes of microorganisms such as E. coli which contains oxygen scavenging membrane fragments, can be used alone or in combination with other adjuvants.

Detail Description Paragraph:

[0066] The preferred Oxyrase.RTM. enzyme system utilized as an adjuvant in the invention is comprised of oxygen scavenging membrane fragments which contain an electron transport system which reduces oxygen to water in the presence of a hydrogen donor. These oxygen scavenging membrane fragments can be derived from the cytoplasmic membranes of bacteria and/or from the membranes of mitochondrial organelles of a large number of higher non-bacterial organisms. Other known biocatalytic oxygen reducing agents such as glucose oxidase, alcohol oxidase, catalase, etc. can also be supplemented or utilized in the present invention, although generally less preferably.

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US Pk Pub 20020045248 A1

Summary of Invention Paragraph:

[0030] The biocatalytic oxygen reducing agents suitable for use in the invention are non-toxic to microorganisms. Being catalysts, they are dynamic and highly efficient at reducing the oxygen in the trapped headspace in the specially designed culture dish. The biocatalytic oxygen

Summary of Invention Paragraph:

[0031] In this regard, the present invention is directed to a specifically designed culture dish with a dish top or cover that contains a sealing ring on the inside upon which the solid media surface in the bottom dish rests when the dish is inverted to form a media-ring seal. The seal so formed traps the gas in the headspace between the media surface and the inside of the dish top or cover. In addition, an oxygen reducing agent, such as a biocatalytic oxygen reducing agent, can be incorporated into the media present in the culture dish together, in some instances, with a substrate which reacts with oxygen in the media and the headspace to create an environment suitable for growing anaerobic microorganisms.

Summary of Invention Paragraph:

[0032] The preferred biocatalytic oxygen reducing agent (see "A Novel Approach to the Growth of Anaerobic Microorganisms" of Adler, et al., Biotechnol. Bioeng. Symp. 11 J. Wiley & Sons, New York, 1981, p. 533 and U.S. Pat. No. 4,476,224 issued Oct. 9, 1984 to Adler entitled "Material and Method for Promoting the Growth of Anaerobic Bacteria") utilized in the invention is comprised of oxygen scavenging membrane fragments which contain an electron transport system which reduces oxygen to water in the presence of a hydrogen donor. These oxygen scavenging membrane fragments can be derived from the cytoplasmic membranes of bacteria (U.S. Pat. No. 4,476,224) and/or from the membranes of mitochondrial organelles of a large number of higher non-bacterial organisms. Other known biocatalytic oxygen reducing agents such as glucose oxidase, alcohol oxidase, etc. can also be utilized.

Summary of Invention Paragraph:

[0033] The biocatalytic oxygen reducing agents suitable for use in the invention are non-toxic to microorganisms. Being catalysts, they are dynamic and highly efficient at reducing the oxygen in the trapped headspace in the specially designed culture dish. The biocatalytic oxygen reducing agents use substrates that are commonly found in microbiological media and that are natural to microorganisms to effect this reaction. The products produced from this reaction are also natural and non-toxic to microorganisms. The use of the biocatalytic oxygen reducing agents makes possible the opening and closing of this dish several times and the agents continue to reduce the oxygen trapped in the headspace after each occurrence.

Summary of Invention Paragraph:

[0037] Furthermore, when the culture dish is utilized with the oxygen reducing agent such as a biocatalytic oxygen reducing agent, the oxygen reducing agent in the media reacts with the oxygen trapped in that headspace. This reaction renders the headspace sufficiently low in oxygen such that microorganisms affected by the presence of oxygen can grow on the media surface typically within 24 to 48 hours when the dish is incubated at 35.degree. C. to 37.degree. C. in an aerobic incubator. Any oxygen that intrudes into the dish around the media ring-seal or through the plastic is removed by the action of the reducing agent. The catalytic reducing agent facilitates the design and function of this dish.

Detail Description Paragraph:

[0082] A variant of the culture dish contains one or more perforations or pores 132 in the dish bottom 12 for the purpose of controlling moisture inside the headspace 80. The sizes of the pores 132 can vary but are usually about one-tenth (0.1) cm to three-tenths (0.3) cm in diameter. The number of pores 132 can vary from one (1) to eighty (80) or more and their location can be grouped or evenly spaced. The pores may be covered with an adhesive film (not shown) such as Mylar.TM. which retards the passage of oxygen and can be sterilized in place when the dish is sterilized.

This film can be removed after the culture dish 10 is filled and before it is incubated. The pores provide a means to reduce the water content of the media during incubation in a controlled manner. This reduces the condensate that forms inside the assembled culture dish 10. Any oxygen infiltrating into the assembled culture dish 10 through these pores 132 must pass through the media 14 to get to the media surface where the microbes 20 are planted. The media 14 contains the biocatalytic oxygen reducing agent and optionally one or more substrates that removes the oxygen before it can reach the surface by this route.

Detail Description Paragraph:

[0095] The biocatalytic oxygen reducing agents suitable for use in the invention include known biocatalytic oxygen reducing agents such as glucose oxidase and catalase and the oxygen scavenging bacterial cell membrane fragments disclosed in U.S. Pat. No. 4,476,224 entitled "Material and Method for Promoting the Growth of Anaerobic Bacteria", issued Oct. 9, 1984 to Howard I. Adler, Oak Ridge, Tenn., one of the co-inventors of the present invention. The '224 patent is incorporated herein by reference.

Detail Description Paragraph:

Growth of Anaerobic Microorganisms Using the Culture Dish, i.e., "OxyDish.TM." of the Present Invention and a Biocatalytic Oxygen Reducing Agent

Detail Description Paragraph:

[0113] Nutrient agar is supplemented with sodium formate (15 mM), sodium succinate (30 mM), sodium lactate (45 mM) and cysteine (0.025 g/100 ml). A biocatalytic oxygen reducing agent, EC-Oxyrase.RTM. (Oxyrase, Inc., Mansfield, Ohio) is added to cooled (45.degree. C. to 50.degree. C.) but molten sterile medium to give a final concentration of 5 units/ml. 20 ml of the above mixture is soon introduced into the bottom part of a culture dish, i.e., "OxyDish.TM.". The top part of the culture dish, is placed over the filled bottom part to prevent contaminants from entering the dish. The agar in the bottom part cools to ambient temperature and solidifies. The covered dish is left standing to permit excess moisture to escape. At this point the dish may be sealed by inverting it to bring the agar surface in the dish bottom into contact with the ring inside the dish top.

Detail Description Paragraph:

[0114] A suspension of anaerobic microorganisms is spread on the surface of the agar medium that contains the biocatalytic oxygen reducing agent and its substrates. The dish is sealed by inverting it. The dish is then placed into an aerobic incubator at 35.degree. C. to 37.degree. C. for 24 to 48 hours. Several dishes are stacked to form a stable column of dishes.

Detail Description Paragraph:

[0116] Using this technique with the culture dish, i.e., "OxyDish.TM." and a biocatalytic oxygen reducing agent, the following microorganisms have been grown:

Detail Description Paragraph:

Measurement of Oxygen Depletion in the Headspace of the Culture Dish Effect of the Present Invention by the Biocatalytic Oxygen Reducing Agent

Detail Description Paragraph:

[0118] A hole is drilled in the base of the culture dish, i.e., "OxyDish.TM." and a gas tight septum is inserted. The base is then filled with 20 ml of agar containing a biocatalytic oxygen reducing agent. The bottom is sealed to the top by inverting the assembled dish and incubating it at 37.degree. C. Periodically, 50 ul samples of the gas in the headspace of the dish are sampled by inserting the tip of a 100 ul gas tight Hamilton syringe through the septum in the base of the dish. These samples are introduced into an Oxygen Sensor (IT Corporation) and the concentration of oxygen remaining in the headspace is determined. Using this method it has been determined that all measurable oxygen, less than 10 pp billion, is removed from the



head space in two to eight hours depending on the concentration and configuration of the biocatalytic agent used. It has also been determined that the dish can be opened, resealed and, after a suitable incubation period, the head space again becomes anaerobic.

Detail Description Paragraph:

Rapid Anaerobiosis of the Agar Layer Containing a Biocatalytic Oxygen Reducing Agent as Indicated by Methylene Blue.

Detail Description Paragraph:

[0121] A small rectangular piece of filter paper impregnated with methylene blue at an alkaline pH is fixed to the inside of the dome in the top of the culture dish, i.e., "OxyDish.TM.". The dish bottom contains nutrient agar and a biocatalytic oxygen reducing agent. The dish is sealed by inverting it thereby causing the agar surface to rest on the ring. After incubation at 37.degree. C. for 8 hours or more, the blue color disappears from the filter paper. This indicates that the headspace of the culture dish, i.e., "OxyDish.TM." has become anaerobic.

Detail Description Paragraph:

Use of Glucose Oxidase and Catalase as the Biocatalytic Oxygen Reducing Agent

Detail Description Paragraph:

[0123] A piece of filter paper saturated with a 1% sodium bicarbonate solution and then dried is fixed to the inside of the dome in the culture dish, i.e., "OxyDish.TM." top. This filter paper is then covered by a 0.2 u membrane filter. The dish bottom is filled with 20 ml of Nutrient Agar (Difco) and a biocatalytic oxygen reducing agent, EC-Oxyrase.RTM. at 5 units/ml and substrates. The agar surface is inoculated by streaking with a small amount of Clostridium acetobutylicum, a microorganism that requires CO.sub.2 for rapid colony development. Immediately before sealing the dish, one drop of 0.1 N HCl is placed on the membrane filter. The dish is sealed by inverting it and placed into a 37.degree. C. aerobic incubator. After 24 hours of incubation, growth of C. acetobutylicum can be observed indicating that CO.sub.2 was released from the sodium bicarbonate impregnated filter paper into the headspace of the culture dish, i.e., "OxyDish.TM.".

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L13: Entry 47 of 64

File: USPT

Jul 31, 2001

DOCUMENT-IDENTIFIER: US 6268481 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Covalently coupled troponin complexes

Detailed Description Text (30):

Anti-microbial and anti-fungal agents may be added to prevent growth and may include those commonly found in the prior art at the concentrations found in the prior art such as gentamycin, clotrimazole, sodium azide, mycostatin, thimerosal, Kathon and/or Proclin 300.

Other Reference Publication (26):

Jacobson, K. Bruce et al. "Partial Purification of an Oxygen Scavenging Cell Membrane Fraction for Use in Anaerobic Biochemical Reactions" Biotechnology and Applied Biochemistry (9) 368-379 (1987).

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PGPUB-DOCUMENT-NUMBER: 20030138867  
PGPUB-FILING-TYPE: new  
DOCUMENT-IDENTIFIER: US 20030138867 A1

TITLE: Medium composition, method and device for selectively enhancing the isolation of anaerobic microorganisms contained in a mixed sample with facultative microorganisms

PUBLICATION-DATE: July 24, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY
Copeland, James C.	Ashland	OH	US
Myers, Kathy J.	Mansfield	OH	US

APPL-NO: 10/007739 [PALM]  
DATE FILED: November 8, 2001

RELATED-US-APPL-DATA:  
non-provisional-of-provisional 60246872 20001108 US

INT-CL: [07] G01 N 33/554, G01 N 33/569, C12 Q 1/04

US-CL-PUBLISHED: 435/7.32  
US-CL-CURRENT: 435/7.32

REPRESENTATIVE-FIGURES: NONE

ABSTRACT:

The present invention is directed to a medium, broth or agar, and a method of utilizing the same, in order to isolate and/or identify anaerobes from a mixed sample that contains facultative microorganisms. The medium contains an inhibitor of the electron transport system, such as a salt of azide (N.sub.3.sup.-), cyanide (CN.sup.-) or related compounds. These inhibitors are present in an amount sufficient to limit the growth of facultative microorganisms under anaerobic conditions while not inhibiting the growth of the anaerobe microorganisms. Preferably, the inhibitor is present in the amount of from about 0.1 mg/ml to about 1.0 mg/ml in broth medium, and from about 0.01 mg/ml to 1.0 mg/ml in agar medium.

[0001] The present application claims the benefit of priority to U.S. Provisional Application Serial No. 60/246,872 filed on Nov. 8, 2000.

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\$AZIDE	0
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IIIAAAZIDE	1
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2-QUINONEDIAAZIDE	1
12-QUINONEDIAAZIDE	1
SULFOIAAZIDE	1
SUPPLY-N-IAAZIDE	1
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12-QUINONEDIAAZIDE	1
SULFOIAAZIDE	1
SUPPLY-N-IAAZIDE	1
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DOCUMENT-IDENTIFIER: US 5633165 A

TITLE: Fermentor with vertical shaft

Detailed Description Text (50):

All bacteria, including both archaeobacteria and eubacteria, generally have more than one terminal oxidase (Anraku and Gennis, supra), and thus all except obligate anaerobes are potentially susceptible to DO.sub.2 instabilities upon culturing. Suitable bacteria for this purpose include aerobic and facultative anaerobic bacteria, whether archaeobacteria and eubacteria, especially eubacteria, and most preferably Enterobacteriaceae. Examples of useful bacteria include Escherichia, Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla, and Paracoccus. Suitable E. coli hosts include E. coli W3110 (ATCC 27,325), E. coli 294 (ATCC 31,446), E. coli B, and E. coli X1776 (ATCC 31,537). These examples are illustrative rather than limiting. Mutant cells of any of the above-mentioned bacteria may also be employed. It is, of course, necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, E. coli, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYA177, or pKN410 are used to supply the replicon.

Detailed Description Text (68):

It has been found herein that when the organism has previously been induced for the presence of menaquinone and the cytochrome d oxidase complex, it has the ability to scavenge low concentrations of oxygen but does so inefficiently. When sufficient oxygen is present, it would appear that this inefficient pathway is not allowed to function.

Other Reference Publication (20):

Doelle and Hollywood, "Transitional Steady-State Investigations during Aerobic-Anaerobic Transition of Glucose Utilization by Escherichia coli K-12" European Journal of Biochemistry 83:479-484 (1978).

Other Reference Publication (32):

Harrison and Loveless, "The Effect of Growth Conditions on Respiratory Activity and Growth Efficiency in Facultative Anaerobes Grown in Chemostat Culture" J. Gen. Microbiology 68:35-43 (1971).

Other Reference Publication (71):

Tamura-Lis and Webster, "Cyanide- and Carbon Monoxide-Resistant Mutants of Vitreoscilla: Altered Cytochromes and Respiratory Properties" Arch. Biochem. and Biophys. 244(1):285-291 (1986).

Other Reference Publication (72):

Tamura-Lis and Webster, "Respiration in Carbon Monoxide and Cyanide Resistant Strains of Vitreoscilla" Fed. Proc. (Ab. #2799) 41(3):749 (1982).

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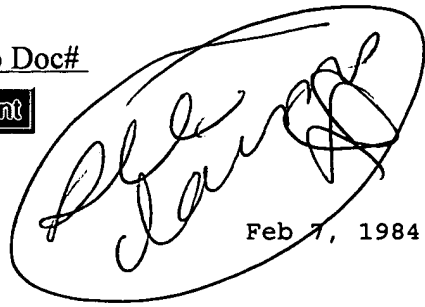
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Feb 7, 1984



DOCUMENT-IDENTIFIER: US 4430427 A

TITLE: Red absorbing combination of alcohol oxidase and an azide compoundAbstract Text (1):

Combining active alcohol oxidase with sufficient amount of an azide compound has been found to form a red absorbing combination. Formation of the red absorbing combination enables determining the presence of active alcohol oxidase by adding the azide compound to a preparation and observing the resultant color. Additionally, alcohol oxidase may be purified by adding the azide compound to a preparation containing active alcohol oxidase in an amount effective to produce a red absorbing complex and separating the red absorbing complex from the preparation.

Brief Summary Text (17):

According to the invention is a composition of matter comprising alcohol oxidase and an azide compound. Further according to the invention is a method of determining the presence of active alcohol oxidase by adding an azide compound, if such a compound is not present, and observing the resulting color. Further according to the invention is a method of purifying alcohol oxidase comprising making an enzyme preparation comprising alcohol oxidase, adding an azide compound to form a light absorbing combination with the alcohol oxidase, and separating the light absorbing combination from the rest of the enzyme preparation.

Brief Summary Text (19):

According to the invention there is provided a composition of matter comprising an azide having the characteristic formula  $R''(N_{\text{sub}3})_{\text{sub}x}$ , where  $R''$  may be a metal atom, a hydrogen atom, the ammonium radical, a complex, and the like, and an enzyme preparation comprising alcohol oxidase.

Brief Summary Text (20):

As indicated, the azide can be any azide which combines with alcohol oxidase to produce a new specifically absorbing species. Preferred azides are the metal salts of azides, particularly the electropositive metal azides which are not explosive. Particularly preferred are metal azides selected from Group 1A of the Periodic Table according to Mendeleev, such as lithium azide, sodium azide, potassium azide, and the like.

Brief Summary Text (36):

The azide compound is added to the alcohol oxidase preparation in an amount effective to produce an absorbance change preferably observed at 522 nm (broadly observed at 300-650 nm) in the presence of active enzyme. The amount of azide to be added can be readily determined by the artisan. Broadly, however, the azide can range from about 1 mole azide ( $N_{\text{sub}3}^{\text{sup} -}$ ) per mole enzyme to about  $5 \times 10^7$  moles azide ( $N_{\text{sub}3}^{\text{sup} -}$ ) per mole enzyme where the lower limit is an amount effective to give a detectable change in absorbance at, for example, 522 nanometers and the upper limit is determined by solubility of the azide ( $N_{\text{sub}3}^{\text{sup} -}$ ) in water. Preferably, the azide is present in an amount in excess of that required to saturate the binding sites of the enzyme, i.e., above about 8 moles azide per mole enzyme as shown in Example XII, for maximum color development. Most preferably the azide is present in an amount in the range of about 10 moles

azide to about 2000 moles azide per mole enzyme.

Brief Summary Text (37):

The amount of azide can also be expressed in weight/weight terms if the molecular weight of the enzyme is known. For the alcohol oxidase isolated from *Pichia pastoris* described herein and having an estimated molecular weight of about 625,000, the azide can be added broadly in an amount in the range of about 0.07 mg to about 80,000 mg per gram alcohol oxidase, more preferably above about 0.1 mg per gram alcohol oxidase, most preferably in the range of about 0.7 mg to about 135 mg azide per gram alcohol oxidase.

Brief Summary Text (38):

The amount of azide to be added can also be expressed in terms of the enzyme activity and this may be particularly convenient when dealing with crude preparations. In the instance of the alcohol oxidase having a specific activity of 10-12 E.U. (enzyme units) per milligram of alcohol oxidase, the azide can be broadly in the range of from about 7.times.10.sup.-6 mg to about 8 mg azide (N.sub.3.sup.-) per Enzyme Unit, preferably above about 7.times.10.sup.-5 mg azide (N.sub.3.sup.-)/EU, most preferably in the range of about 7.times.10.sup.-4 mg azide (N.sub.3.sup.-) /EU to about 1.35.times.10.sup.-2 mg azide (N.sub.3.sup.-)/EU. By an enzyme unit (EU) as used herein is meant the amount of enzyme required to oxidize 1 .mu.mole MeOH in one minute. Correlative ranges for other alcohol oxidases having other specific activities can be readily determined by the artisan.

Brief Summary Text (40):

As indicated, the invention also comprises methods of purifying alcohol oxidase comprising preparing an enzyme preparation comprising alcohol oxidase, adding an azide compound effective to form a light absorbing combination with the alcohol oxidase, and separating the light absorbing combination from the rest of the enzyme preparation. The enzyme preparation can be prepared by any suitable preparative method such as, for example, those set forth above. The azide can be added also as set forth above. The separating step can use any suitable procedure for separating the red absorbing combination from the other components present in the crude preparation. The red absorbing combination can be removed, for example, (1) by differential solubility using, for example, ammonium sulfate or polyethylene glycol; (2) by specific precipitation; (3) by column chromatography; (4) by preparative electrophoresis; (5) by preparative ultracentrifugation; (6) by any other enzyme preparative method known to the artisan; or (7) by dialysis to crystallization as herein described. Other suitable methods can also be employed by the artisan. Particularly preferred are those methods such as column chromatography, electrophoresis and the like where the color absorbing complex can facilitate recovery of purified protein.

Detailed Description Text (26):

This example demonstrates the color-forming interaction of alcohol oxidase with sodium azide. Color is observed within a few seconds, and persists.

Detailed Description Text (29):

Fermentation effluent from the aerobic fermentation of *Hansenula polymorpha* (prepared as described in Example II) was adjusted to pH 7.5 with concentrated ammonium hydroxide. Sample was homogenized and disrupted on a Dyno-Mill Model KDL using a 0.6 L vessel in a continuous operation at 30.degree. C. using belt combination #3 and a flow of 20-30 mL/hr. The beads were lead-free glass beads with a diameter of 0.3-0.5 mm. The resulting homogenate was centrifuged for 20 minutes at 10,000.times.g to yield a cell-free supernatant. The material thus prepared exhibited a specific activity of 4.9 (when analyzed by the dye-peroxidase method described in Example I), and gave a red color when treated with sodium azide as follows:

Detailed Description Text (34):

Hansenula polymorpha cell homogenate (prepared as described in Example II) (150 mL) is dialyzed against water to remove salts and other low molecular weight materials, then subjected to sequential protein precipitation by treatment with increasing amounts of polyethylene glycol (PEG) with a molecular weight of 6000. Sample is allowed to settle for 15 minutes after addition of PEG, then centrifuged at 12,000.times.g for 30 minutes. The resultant pellet is resuspended in 150 mL standard phosphate buffer (pH -7.5) and assayed for alcohol oxidase activity as described in Example I and color formation upon addition of sodium azide crystals. The supernatant liquid is treated with additional PEG for 15 minutes, and so on, as above. The results are shown in Table 5A:

Detailed Description Text (36):

This example demonstrates that a qualitative correlation exists between alcohol oxidase activity in different fractions and the formation of red color in the presence of azide ion.

Detailed Description Text (40):

This example demonstrates that treatment to denature the enzyme alcohol oxidase to produce inactive alcohol oxidase causes loss of the enzyme-azide red color.

Detailed Description Text (43):

Into an anaerobic cuvette is placed 2.5 mL of purified Pichia alcohol oxidase (purified as described in Example I) with a few crystals of sodium azide (about 1 mg). The absorption spectrum is scanned from 350-550 nm, with particular attention to absorption maxima at 370-380 nm and 450-470 nm. This spectral region includes the absorption bands characteristic of the oxidized form of flavine adenine dinucleotide (FAD) cofactor (Scan 1). An excess of oxygen-free ethanol is then injected into the cuvette, and the sample is again scanned from 350-550 nm (Scan 2). The optical densities of the resultant absorption maxima at about 380 and 450 nm are tabulated below. There is a substantial decrease in the absorption at the wavelengths characteristic of oxidized cofactor, FAD. The color of the sample changes from red to pale yellow upon introduction of ethanol. When oxygen is reintroduced to the enzyme-alcohol mixture by opening the cuvette to the air, the red color returns. The absorption spectrum is again recorded between 350-550 nm (Scan 3). The data are tabulated in Table 7A below.

Detailed Description Text (47):

Three mL of a purified Pichia alcohol oxidase solution containing about 7 mg/mL enzyme are placed in a cuvette with a 1 cm. pathlength. Sample is then scanned from 350-550 nm (Scan 1). To the sample, in the absence of air, is added 1 .mu.L ethanol. Sample is then scanned again (Scan 2). Maintaining the sample in the absence of air, several crystals (about 1 mg) of sodium azide are mixed with the alcohol-enzyme solution, which is scanned from 350-550 nm again (Scan 3). The cuvette is then opened to the air and stirred. Red color appears for a few seconds, but does not persist for sufficient time to allow a complete scan. Sample simply reverts to the pale yellow observed before air is introduced. Scan 4 records the sample after exposure to air. The results are tabulated in Table 7B.

Detailed Description Text (59):

Since the specific activity of Pichia pastoris alcohol oxidase is known to be 10-12 EU/mg (see Example I), the above data indicate a lower limit of about 0.3 mg alcohol oxidase/mL for visual detection (by eye) of alcohol oxidase by red color formation in the presence of sodium azide.

Detailed Description Text (62):

In a typical application of the red complex of alcohol oxidase-sodium azide, Pichia alcohol oxidase is tested for denaturation by a variety of inorganic salts, as described below. A few drops of crude enzyme (crude homogenate prepared as described in Example I) are placed on a spot plate. Then, sufficient salt crystals

were added to produce a saturated solution. Samples were assayed 1-2 minutes later for color formation and for enzyme activity as described in Example I. The results are tabulated in Table X.

Detailed Description Text (76):

Since about 7.6 moles of azide were found to bind with each mole of enzyme, this example suggests, within experimental error, that one azide molecule binds to each alcohol oxidase subunit.

Detailed Description Text (77):

As thus set forth above in illustrative embodiments and examples, it has been found that active alcohol oxidase changes color upon addition of azide compounds. The color changes are characteristic of the state of the enzyme and a red color is characteristic of active enzyme and is not characteristic of inactive enzyme. This unique color marker makes purification and visual estimation of activity and enzyme concentration very convenient. The invention is not to be limited by the illustrative embodiments and examples herein provided, however, but by the claims appended hereto.

CLAIMS:

1. A composition of matter comprising active alcohol oxidase; and

an azide compound selected from the group of compounds having the formula  $R(N_{\text{sub}3})_{\text{sub}x}$  wherein R is a metal atom, a hydrogen atom, or the ammonium radical, and  $N_{\text{sub}3}$  is the moiety N.dbd.N.dbd.N,

the azide compound being present in an amount effective to form a red absorbing combination with active alcohol oxidase.

3. A composition as in claim 1 wherein:

the azide is present in an amount in the range of about 1 mole azide/mole alcohol oxidase to about  $5.0 \times 10^{0.7}$  moles azide/mole alcohol oxidase.

4. A composition as in claim 1 wherein:

the azide is present in an amount in the range of about 8 moles azide/mole alcohol oxidase to about  $5.0 \times 10^{0.7}$  moles azide/mole alcohol oxidase.

5. A composition as in claim 1 wherein:

the azide is present in an amount in the range of about 10 moles azide/mole alcohol oxidase to about 2000 moles azide/mole alcohol oxidase.

6. A composition as in claim 1 wherein:

the alcohol oxidase is isolated from genus Pichia; and

the azide is present in an amount in the range of about 0.07 mg azide/gram alcohol oxidase to about 80,000 mg azide/gram alcohol oxidase.

7. A composition as in claim 1 wherein:

the alcohol oxidase is isolated from genus Pichia; and

the azide is present in an amount in the range of about 0.1 mg azide/gram alcohol oxidase to about 80,000 mg azide/gram alcohol oxidase.

8. A composition as in claim 1 wherein:

the alcohol oxidase is isolated from genus *Pichia*; and

the azide is present in an amount in the range of about 0.7 mg azide/gram alcohol oxidase to about 135 mg azide/gram alcohol oxidase.

12. A method of purifying an active alcohol oxidase comprising:

adding an azide compound selected from the group of compounds having the formula  $R''(N_{sub.3})_{sub.x}$  wherein  $R''$  is a metal atom, a hydrogen atom, or the ammonium radical, and  $N_{sub.3}$  is the moiety  $N.dbd.N.dbd.N$  to a preparation comprising the active alcohol oxidase in an amount effective for producing a red absorbing complex; and separating the red absorbing complex from the preparation.

13. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 1 mole azide/mole alcohol oxidase to about  $5.0 \times 10^{sup.7}$  moles azide/mole alcohol oxidase.

14. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 8 moles azide/mole alcohol oxidase to about  $5.0 \times 10^{sup.7}$  moles azide/mole alcohol oxidase.

15. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 10 moles azide/mole alcohol oxidase to about 2000 moles azide/mole alcohol oxidase.

16. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 0.07 mg azide/gram alcohol oxidase to about 80,000 mg azide/gram alcohol oxidase; and wherein

the alcohol oxidase is isolated from genus *Pichia*.

17. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 0.1 mg azide/gram alcohol oxidase to about 80,000 mg azide/gram alcohol oxidase; and wherein

the alcohol oxidase is isolated from genus *Pichia*.

18. A method as in claim 12 wherein adding an azide comprises:

adding an amount of sodium azide in the range of about 0.7 mg azide/gram alcohol oxidase to about 135 mg azide/gram alcohol oxidase; and wherein

the alcohol oxidase is isolated from genus *Pichia*.

28. A method of determining the presence of active alcohol oxidase comprising:

observing the color of a composition of matter comprising an alcohol oxidase and an azide compound selected from the group of compounds having the formula  $R''(N_{sub.3})_{sub.x}$  wherein  $R''$  is a metal atom, a hydrogen atom, or the ammonium radical, and  $N_{sub.3}$  is the moiety

$N.dbd.N.dbd.N$

the azide compound being present in an amount effective to form a red absorbing

combination with active alcohol oxidase.

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Am J Physiol. 1983 Mar;244(3):R356-62.

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**Atlantic hagfish cardiac muscle: metabolic basis of tolerance to anoxia.****Hansen CA, Sidell BD.**

Oxygen tensions in the major venous inputs to the systemic and portal-vein hearts of normoxic Atlantic hagfish ( $12.3 \pm 1.7$  and  $11.0 \pm 1.6$  mmHg, respectively) are low compared with typical vertebrate values. Anoxia and poisoning with cyanide and azide do not significantly affect in situ performance of the systemic heart. Idoacetate poisoning, however, results in a significant decrease in cardiac performance of the systemic heart to 12% of the initial value after 3 h. Activities of mitochondrial enzymes of hagfish ventricle suggest a small potential for aerobic metabolism compared with those in the aerobic ventricle of Atlantic cod. Activities of enzymes of carbohydrate metabolism indicate similar anaerobic capacity in hagfish and cod ventricle. The ratio of pyruvate kinase to cytochrome c oxidase, an index of anaerobic to aerobic capacity, is 5.6 times greater in hagfish than cod ventricle. Metabolite concentrations in freeze-clamped ventricles of normoxic and hypoxic hagfish indicate hypoxia-induced activation of glycogenolysis, enhanced substrate flow across 6-phosphofructokinase, and an apparent secondary constriction of glycolysis at the level of glyceraldehyde-phosphate dehydrogenase. Carbohydrate utilization via the glycolytic pathway appears essential for maintenance of cardiac performance in both normoxic and anoxic hagfish. Under conditions of severe hypoxia, ATP provision is probably met by anaerobic glycolysis.

PMID: 6299122 [PubMed - indexed for MEDLINE]

Am J Physiol. 1993 Apr;264(4 Pt 1):C961-7.

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## Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes.

Dawson TL, Gores GJ, Nieminen AL, Herman B, Lemasters JJ.

Department of Cell Biology and Anatomy, School of Medicine, University of North Carolina, Chapel Hill 27599-7090.

Cell killing, oxygen consumption, and hydroperoxide formation were determined in rat hepatocytes after glycolytic and respiratory inhibition. These conditions model the ATP depletion and reductive stress of anoxia ("chemical hypoxia"). Glycolysis was inhibited with iodoacetate, and mitochondrial electron transfer was blocked with sodium azide, cyanide, or myxothiazol. Cell killing, hydroperoxide formation, and inhibitor-insensitive oxygen consumption were greater after azide than after myxothiazol or cyanide. Desferrioxamine, an inhibitor of iron-catalyzed hydroxyl radical formation, delayed cell killing after each of the respiratory inhibitors. Anoxia also delayed cell killing during chemical hypoxia. However, during anoxic incubations, desferrioxamine did not delay the onset of cell death. These findings indicate that reactive oxygen species participate in lethal cell injury during chemical hypoxia. In isolated mitochondria, previous studies have shown that myxothiazol inhibits Q cycle-mediated ubisemiquinone formation in complex III (ubiquinol-cytochrome c oxidoreductase) and that ubisemiquinone can react with molecular oxygen to form superoxide. Decreased killing of hepatocytes with myxothiazol compared with azide suggests, therefore, that mitochondrial oxygen radical formation by complex III is involved in cell killing during reductive stress. In support of this hypothesis, myxothiazol reduced rates of cell killing and hydroperoxide formation in hepatocytes incubated with azide or cyanide. This mitochondrial mechanism for oxygen radical formation may be important in relative but not absolute hypoxia.

PMID: 8386454 [PubMed - indexed for MEDLINE]

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Eur J Biochem. 1993 Apr 1;213(1):563-71.

Related Articles, Links

**Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) from a denitrifying *Pseudomonas* species.****Brackmann R, Fuchs G.**

Angewandte Mikrobiologie, Universitat Ulm, Germany.

The reductive removal of aromatic hydroxyl functions plays an important role in the anaerobic metabolism of many phenolic compounds. We describe a new enzyme from a denitrifying *Pseudomonas* sp., 4-hydroxybenzoyl-CoA reductase (dehydroxylating), which reductively dehydroxylates 4-hydroxybenzoyl-CoA to benzoyl-CoA. The enzyme plays a role in the anaerobic degradation of phenol, 4-hydroxybenzoate, p-cresol, 4-hydroxyphenylacetate, and other aromatic compounds, of which 4-hydroxybenzoyl-CoA is an intermediate. The enzyme is therefore induced only under anoxic conditions with these aromatic substrates, but not with benzoate or under aerobic conditions. A similar enzyme which reductively dehydroxylates 3-hydroxybenzoyl-CoA is induced during anaerobic growth with 3-hydroxybenzoate. The soluble enzyme 4-hydroxybenzoyl-CoA reductase was purified. It has a molecular mass of 260 kDa and consists of three subunits of 75, 35, and 17 kDa. The subunit composition is likely to be  $\alpha_2\beta_2\gamma_2$ . The enzyme contains 12 mol iron/mol and 12 mol acid-labile sulfur/mol and exhibits a typical ultraviolet/visible spectrum of an iron-sulfur protein. The reaction requires a reduced electron donor such as reduced viologen dyes; no other co-catalysts are required, the product is benzoyl-CoA and oxidized dye. The reductase is rapidly inactivated by oxygen. The inactivation by low concentrations of cyanide or azide in a pseudo-first-order time course suggests that it may contain a transition metal in an oxidation state which reacts with these ligands. 4-Hydroxybenzoyl-CoA reductase represents a type of enzyme which is common in anaerobic aromatic metabolism of phenolic compounds. A similar enzyme is demonstrated in *Rhodopseudomonas palustris* anaerobically grown with 4-hydroxybenzoate. The biological significance of reductive dehydroxylation of aromatics and a possible reaction mechanism similar to the Birch reduction are discussed.

PMID: 8477729 [PubMed - indexed for MEDLINE]